Proposed Title for Ph.D. Research Work

Anthelmintic assessment of *Clerodendrum infortunatum* Linn.

on fish parasites

Name of the candidate

Netri Datta

Name of the guides

1. Prof. Samar Kumar Saha

Department of Zoology

Visva-Bharati University, Santiniketan

2. Prof. Pradip Kumar Kar

Department of Zoology

Coochbehar Panchanan Barma University

Registration No.

VB-1721 of 2015-2016

Date of admission

29/08/2015

Introduction:

Aquaculture of fin fish is an important economic activity throughout the world. One of the major problemsaffecting freshwater fishproductionis associated with various parasitic diseases. As a strategy of management of parasitic infection several synthetic chemical substances such as, praziquantel, toltrazuril, and mebendazoleare used as therapeutics(Schmahl and Melhorn, 1988). But if thoseare not applied properly, may result hazardous effect on the environment and become toxic to the fishes, fishery staffs and consumers. Besides, the frequent application of these chemicals show limited efficacy by developing drug-resistant parasites. Other chemicals, such as trichlorfon and formalin, are also far from being satisfactory (Goven and Amend, 1982). Therefore, now-a-days application of the plant derived products is becoming a better alternative methodfor removal of parasites,So far the environmental hazard is concerned, toxicity of phytochemicals is quite low and degradability is faster compared with that of the synthetic pesticides. There is a number of plant derived compounds whose antiparasitic efficacy are proven through research experiments. Many of those have therapeutic potentials and are the source of bioactive and low cost compounds. This can be applied as pharmaceutical products against parasites without harming the environment(Knaak and Fiúza, 2010).

Among the metazoans, helminths comprise a large group of fish parasite.There are several monogenean,digenean, cestodeand nematode parasites which cause infections among the fishes. Some of them are *Dactylogyrus*sp., *Gyrodactylus* sp., *Ancyclodiscoides* sp., *Cardicola* sp., *Diplostomum* sp., *Gangesia*sp. etc. The most effective therapeutic agent against those helminths is formaldehyde. Though it exhibits high success rate to remove the helminth parasites from fish, it is classified as carcinogenic by the World Health Organization (2006). On the other

hand,different synthetic drugs frequently used asanthelmintics, such as, praziquantel, toltrazuril, levamisole, trichlorfon, and several benzimidazole derivatives etc. are proven not ideal for applying on fishes due to causing toxicity on them, when giving at high concentration and not effecting properly when applying in low concentration . Applications of these common drugs are proved dangerous to aquatic environment, especially in tropical climate. Organophosphates, such as, trichlorfon are not generally used because they cause irreversible effects in nontarget species by phosphorylating acetylcholinesterase (Kozlovskaya and Mayer,1984; Pena et al., 2003; Costa, 2006).Another compound such as benzimidazole is known tocause toxicity in the aquatic ecosystem (Oh et al., 2006). Moreover, resistance of thehelminth parasites against such products has been reported (Schmahl et al, 1989;Isaacs, 1983).Furthermore, those are not only toxic to the fishes, but also to the zooplanktons, phytoplanktons and human consumers.

Owing to these difficulties it is imperative to find out suitable therapeutic substances against helminth diseases of fish asbetter alternatives.The natural products from plants could be the better alternativeantihelminthics with respect toefficacy, environmental compatibility and fish health perspective. With these rationales the proposed plan of research work has been designed keeping the following objectives in mind.

National and international status:

Several works had been performed by the scientists to show the potentiality of the plant derived compounds as anthelmintics on fish helminth parasites throughout the world. In vitro and oral administration trials were done by some scientists for finding effective plant derived compounds against infection by monogenean *Heterobothrium okamotoi* in the tiger puffer fish (*Takifugu rubifes*) (Hirazawa et al.,2000). They experimentally proved that caprylic acid can be used as a potent natural anthelmintic. Eugenol, an active component of several plants, particularly *Eugenia*

caryphylla(Isaacs, 1983) and *Ocimum gratissium* (Silva et al., 2012), was proven to be a non toxic plant derived anthelmintic substance, when it was experimentally used on tambaqui (*Colossoma macropomum*), infected by monogenean ectoparasites (Camurca et al., 2005).In the experiment, eugenol showed effective antiparasitic activity for reduction of the monogenean load and exhibit good activity in the inhibition of nematode egg hatching and as well as in vitro adult helminthic control. Osthole, the active compound of *Radix angelicae pubescentis* was applied against *Dactylogyrus intermedius* infected in gold fish in vivo (Wang et al., 2010). By that experiment, it was concluded that osthol may be effective and safe for controlling *Dactylogyrus* in goldfish. Arctigenin and arctiin, the active compounds from another plant, *Fructus arctii*, also proved to be potential anthelhintic natural drug against the same parasite *D. intermideus* in goldfish (Wang et al., 2009). Many other medicinal plants were used by a number of scientists for this purpose. Such plants are *Dryopteris crassirhizoma, Caesalpinia sappen, Lysima chiachristinae, Cuscuta chinensis, Artemisia argyi, Eupatorium fortunei, Abrus cantoniense, Citrus medica, Dioscorea collettii, Polygonum multiflorum, Santalum album, Chelidonium majus, Macleaya microspora*etc. (Jiang et al., 2013; Huang et al., 2013; Hu et al., 2014; Tu et al., 2013; Yao et al.,2011; Wang et al., 2010). Apart from these medicinal plants, other common natural products, such as lime, onion, garlic etc can be used for alternative antiparasitic treatment. As an example, it was reported that the fish diet, containing garlic, showed anthelmintic property in cultivated *Piaractus mesopotanicus* fishes against *Anacanthorus penilabiatus* (monogenea) infection (Martins et al., 2012).

Objectives:

- Selection of plant derivatives.
- Determination of effective dose.
- Evaluation of efficacy of the plant-derivatives onsome helminth parasites of fish in vivo and in vitro conditions.
- Biochemical analysesof the helminth parasites after exposure to experimental doses of plant derived compound with special emphasis on assessment of tegumental and gastro intestinal enzymes.
- Histochemical analyses of various components of digestive system of the helminth parasites, after treating them with the plant-derived phytoproducts.
- Assessment of efficacy of the plant derivatives at ultrastructural organization level to underscore the mode of action of the compound.

Selection of plants with anthelmintic properties:

Three different plants could beselected for experimentation depending upon some novel properties of the active ingredients present, availability of the plant etc.

***Clerodendrum* sp.:**

Family – Lamiaceae

Distribution – India, Sri lanka, Thailand and Europe

Parts used for medicinal purpose – Root, leaf

Previous works with the plant–A group of scientists concluded that the crude extract of roots of *C. eriophyllum* has antiparasitic activity against *Staphylococcus aureus*, *Cryptococcus neoformans* and *Leishmania donovani* by their experiments (Machumi et al., 2010).

The crude leaf extract of *C. colebrookianum* was experimentally proven to have anthelmintic efficacy against *Hymenolepis diminuta* (Yadav, 2012).

Plan of work:

Collection of plant materials:

The required parts of the selected plants will be collected from local areas or from botanical gardens.

Preparation of crude extracts:

The plant parts will be washed by distilled water. Then they will be air-dried under sunlight for some days. Then they will be finally oven-dried at 45°C for 48 hours. After that it will be crushed to produce fine powder. The powdered sample will be freeze-dried to ensure a complete removal of water. Now, a known weight of the plant product will be immersed in rectified spirit (100 g/litre) for 1 – 2 weeks. The solution will be refluxed for 8 hours at 60°C and the suspension will be discarded. This process will be repeated for 3 – 4 times. Then the solution will be filtered through a clean and fine cloth and then through Whatman filter paper. Next, the rectified spirit solvent will be distilled for getting the semisolid crude extract of the plant portions. Then it will be weighed and kept at 4°C (Tandon et al., 1997).

Extraction of active compound:

The prepared alcoholic crude extract will be mixed with hexane and the supernatant solution will be discarded. This process will be repeated for 20 times. Now the extract will be reduced in volume through distillation and passed through silica gel column using hexane, benzene and ethyl acetate as solvents. The column will be prepared using hexane and benzene mixture (6:4) and silica gel of 100-200 mesh. The samplewill be mixed with silica gel of 60-120 mesh and loaded on top of the column. Elution will be done with hexane : benzene mixture at the ratio of 6:4, 5:5, 4:6, … to pure benzene and thereafter with benzene : ethyl acetate at the ratio of 9:1, 8:2, 7:3, … to pure ethyl acetate.The eluted solution will be concentrated through distillation in water bath (Tandon et al., 1997). Now this product will be used for treatment.

In vitro treatment:

The fish helminths will be collected in a gentle way and kept in saline solution in plates and temperature will be maintained at 20°C. For checking the anthelmintic efficacy of the crude extract, multiple concentrations will beapplied to determine their effects on parasites'responsiveness.A particular concentration will be used with three replicates, each containing five parasites. Praziquantel will be used as positive control, whereas, distilled water will be used as negative control.The experimental plates will be incubated at 20 °C, andregularly checked for the number of responsive helminths (Hardy-Smith et al., 2012).The time taken for paralysis and death of the parasites will be noted by observing immobilization of the parasites. The death of those parasites will be confirmed by adding slightly warm water, which will cause response in the helminths, if those will still alive.

In vivo treatment:

The infected fishes will be kept in glass aquaria. The derived plant extract will be dissolved in 1 ml DMSO and made up to 100 ml with distilled water which will be used for the preparation of the different concentrations of plant extracts. Initial tests were conducted to get amoderate concentration range to avoid the mortalityof fish at high concentrations. Each concentration will be applied with triplicate. A negative control will be made by using distilled water,containing the same amount of DMSO as the test samples. Mebendazole will be used as a positivecontrol. The water temperature will be maintained at 23±1°C and the pHranged from 7.0 to 7.5. Mortality of fish will be recordedthroughout the experiments, during which, no food will begiven to the fish.The effectiveness of each treatment will be confirmedby comparison of the parasite number in eachtreatment group with those in the control group after 48 hours treatment (Wang et al., 2009). Finally, anthelmintic efficacy of each treatmentand the positive control group will be calculated usingthe following equation (Wang et al., 2008):

$$\mathbf{AE = [B – T(P)] \times 100\% / B}$$

Where, AE = Anthelmintic Efficacy,

B = Mean number ofsurviving parasites in the negative control,

P = Mean number ofsurviving parasites in the positive control, and

T =Mean number ofsurviving parasites in the treatment.

The parasites, which will be paralyzed by only the most effective plant extract,will be selected to perform SEM, histochemical and biochemical analysis.

Scanning Electron Microscope (SEM):

The parasite will be primarily fixed in 3% glutaraldehyde at 4°C for 24 hours and washed in PBS. Then it will be secondarily fixed in additive fixative (usually, osmium tetraoxide) for 2 hours and repeatedly washed in PBS. Thereafter it will be gradually dehydrated with ascending grades of ethanol. Then the material will be critical point dried using liquid carbon dioxide to avoid liquid-air interface. The dried material will be put on the metal stub and coated with fine layer of gold by the sputter coater machine. The gold coated material will be observed and photographed using a JEOL, JSM-6300 SEM at electron accelerating voltage ranging between 10-20 kV.

Histochemical analysis of some tegumental and gastrointestinal enzymes:

Non-specific esterases (NSE)

The live specimens will be washed in 0.9% PBS and fixed overnight in 10% NBF at pH 7.0 at 4°C. Staining for NSE will be performed according to the method of Holt and Withers (Holt and Withers, 1952).

Cholinesterase (ChE)

Staining for ChE will be done by Gomori's Method (1952)[114] as described by Rahemo and Gorgees (Rahemo and Gorgees, 1987), where the specimens have to be fixed in cold formalin for overnight and processed for staining.

Acetylcholinesterase (AChE)

For the distribution and localization of AChE activity, the frozen cross sections of the material will be fixed in cold-formalin (at 4°C) overnight. The sections will be then processed following

the method of Gomori (Gomori, 1952) as described by Pearse (Pearse, 1968), using standard incubation medium.

Nitric oxide synthase (NOS)

The specimens are to be immersion-fixed in freshly made 4% paraformaldehyde in 0.1 MPBS (pH 7.4) overnight at 4°C. After fixation the parasites will be transferred to PBS with 10% sucrose for some days at 4°C. They will be then embedded in Tissue-Tek, frozen and sectioned sagitally and transversally at 15-20 μm on a cryostat, collected on chrome-alum gelatine/poly-l-lysine coated glass slides, dried for about 2 hours at room temperature and stained directly following Tandon et al. (Tandon et al., 2001). A stock solution of the incubation medium will be made.

Acid phosphatase (AcPase)

The AcPase activity will be observed by the modified lead nitrate method of Takeuchi and Tanoueas described by Pearse(Pearse, 1968), where,β-glycerophosphate are to be used as the substrate.

Alkaline phosphatase (AlkPase)

A modified coupling azo-dye method described by Pearse(Pearse, 1968), will be used for the determination of AlkPase activity.

Adenosine triphosphatase (ATPase)

For observing the ATPase activity, calcium method of Maengwyn-Davies et al., as described by Pearse (Pearse, 1968),will be performed.

5’-Nucleotidase (5’-Nu)

5’-Nu activity will be studied according to the lead method of Wachstein and Meisel (Wachstein and Meisel, 1957)using adenosine monophosphate (AMP) as the substrate.

Biochemical analyses of some tegumental and gastrointestinal enzymes:

Acetylcholinesterase (AChE)

The enzyme activity will be assayed by the method of Ellman et al. (1961), as given by Ott et al. (Ott et al., 1975), where,thiocholine reacts with dithiobisnitrobenzoic acid, which can be estimated colorimetrically.

Nitric oxide synthase (NOS)

The NOS activity will beshown according to the method of Salter and Knowles (Salter and Knowles, 1998).

Acid phosphatase (AcPase)

The AcPase activity will be assayed by estimating the p-nitrophenol product on the basis of the method of Plummer (Plummer, 1988).

Alkaline phosphatase (AlkPase)

The AlkPase activity will beanalysedby the method of Plummer (Plummer, 1988).

Adenosine triphosphatase (ATPase)

The ATPase activity will beshown by estimating the free phosphate released, according to Kaplan’s method (Kaplan, 1957: Rao, 1925).

5’-Nucleotidase (5’-Nu)

The 5'Nu activity will be estimated by the free phosphate according to the method of Bunitian (Bunitian, 1970).

The necessary modifications of the mentioned methods may be done dueto the requirements of experiment.

Expected outcome:

Some alterations may be found in the activity of enzymes after treatment with the plant extracts. Then it will be concluded whether the enzymes of the parasite are the target of action of the plant derived compounds.

Future Prospect:

This project may be able to find novel plant-derived compounds as potential anthelmintic. Furthermore, the histochemical and biochemical analysis will help to find the scientific basis of the action of the phytoproducts on the helminths. Thus, many more vital researches on this regard can be performed. It will help us to avoid the using of synthetic drugs and applying phytoproducts instead of those, so that, we can protect the aquatic environment from the adverse effect of synthetic drugs.

Keywords: Fish helminth parasites, anthelmintic property , plant-derivatives, tegumental enzymes, gastrointestinal enzymes

Literature Cited:

Ayaz M., Junaid M., Subhan F., Ulaah F., Ahmad S., Sadiq A., Imran M., Kamal Z.,Hussain S., Shah S.M.(2014). Heavy metals analysis, phytochemical, phytotoxic and anthelmintic investigations of crude methanolic extract, subsequent fractions and crude saponins from Polygonumhydropiper L. BMC Complementary and Alternative Medicine. 14: 465.

Bulbul L., Jahiruddin Md., Majumder S.S., Roy J.(2013). Phytochemical Screening, Anthelmintic and Antiemetic Activities of *Polygonumlapathifolium* Flower Extract. European Journal of Medicinal Plants. 3(3): 333-344.

Bunitian, H.C. (1970). Deamination of nucleotides and the role of their deamino forms in ammonia formation from amino acids. In: Handbook of Neurochemistry, Lajtha, A (ed.).Plenum, New York.

Camurça-Vasconcelos A.L.F., Morais S.M., Santos L.F.L., Rocha M.F.G., Bevilaqua C.M.L. (2005). Validação de plantasmedicinais com atividade anti-helmíntica.RevistaBrasileira de PlantasMedicinais. 7: 97–106.

Costa L.G. (2006). Current issues in organophosphate toxicology.ClinChimActa. 366:1–13.

Gomori G. (1952). Microscopic histochemistry: Principles and Practice. University of Chicago Press, Chicago.

Goven B.A., Amend D.F. (1982). Mebendazole/trichlorfoncombination: a new anthelmintic for removing monogenetic trematodes from fish. Fish Biology. 20(4): 373-378.

Hardy-Smith P., Ellis D., Humphrey J., Evans M., Evans D., Rough K,ValdenegroV.,Nowak B..(2012).In vitro and in vivo efficacy of anthelmintic compounds against blood fluke (*Cardicolaforsteri*). Aquaculture. 334–337: 39–44.

Hirazawa N, Ohtaka T, Hata K. (2000) Challenge trials on the anthelmintic effect of drugs and natural agents against the Monogenean*Heterobothriumokamotoi*in the tiger puffer *Takifugurubripes.*Aquaculture188: 1–13.

Holt S.J., Withers P.G.J. (1952). Cytochemical localization of esterase using indoxyl derivatives. Nature. 170: 1012-1014.

Huang A.G., Yi Y.L., Ling F., Lu L., Zhang Q.Z., Wang G.X. (2013). Screening of plant extracts for anthelmintic activity against *Dactylogyrusintermedius* (Monogenea) in goldfish (*Carassiusauratus*). Parasitology Research. 112:4065–4072.

Hu Y., Ji J., Ling F., Zhang Q.Z., Wang G.X. (2014). Screening Medicinal Plants for Use against *Dactylogyrusintermedius*(Monogenea) Infection in Goldfish**.** Journal of Aquatic Animal Health26:127–136.

Isaacs G.(1983). Permanent local anaesthesia and anhidrosis after clove oil spillage. Lancet. 1: 882.

Jiang B., Chi C., Fu Y.W., Zhang Q.Z., Wang G.X. (2013). In vivo anthelmintic effect of flavonolrhamnosides from *Dryopteriscrassirhizoma* against *Dactylogyrusintermedius* in goldfish (*Carassiusauratus*). Parasitology Research. 112:4097–4104.

Kaplan C. (1957). Methods in Enzymology. Vol. III. Academic Press, New York. Fiske, CH.

Knaak, N., Fiúza, L.M., 2010. Potencial dos óleosessenciais de plantas no controle deinsetos e microrganismos. Neotropical Biology Conservation. 5: 120–132.

Kozlovskaya V.I., Mayer F.L. (1984) Brain acetylcholinesterase and backbone collagen in fish intoxicated withorganophophate pesticides. Journal of Great Lakes Research. 10:261–266.

Machumi F., Samoylenko V., Yenesew A., Derese S., Midiwo J.O., Wiggers F.T., Jacob M.R., Tekwani B.L., Khan S.I., Walker L.A., Muhammad I. (2010). Antimicribial and AntiparasiticAbietaneDiterpenoids from the Roots of *Clerodendrumeriophyllum*. Natural Product Communications. 5: 853-858.

Majumder M.S., Amin M.N., Moghal M.M.R., Banik S., Kar A., Hossain M.M. (2014). Anthelmintic and Cytotoxic Activities of Two Medicinal Plants: *Polygonumviscosum*and *Aphanamixispolystachya*Growing in Bangladesh. Journal of Scientific Research. 6(2): 339-345.

Martins M.L., Moraes F.R., Miyazakid M.Y., Brum C.D., Onaka E.M., Enerick Jr. J., Bozzo F.R. (2012). Alternative TreatmentFor *Anacanthoruspenilabiatus*(Monogenea: Dactylogyridae) Infection in Cultivated Pacu, *Plaractusmesopotamicus*(Osteichthyes: Characidae) in Brazil and Its Haematological Effects. Parasite. 9: 175-180.

Muddathir A.K., Balansard G., Timon-David P., Badadjamian A., Yagoub A.K., Julien M.J. (1987). Anthelmintic Properties of *Polygonumglabrum*. Journal of Pharmacy and Pharmacology. 39: 296-300.

Oh S.J., Park J, Lee M.J., Park S.Y., Lee J.H., Choi K. (2006.). Ecologicalhazard assessment of major veterinary benzimidazoles:acute and chronic toxicities to aquatic microbes andinvertebrates. Environmental Toxicology and Chemistry. 25:2221–2226.

Ott P., Jerry B., Broadback V. (1975). Multiple molecular forms of purified human erythrocyte acetylcholinesterase. Environmental Journal of Biochemistry. 57: 469-480.

Padmavathi D., Suseela L., Bharathi R.V. (2010). Antidiarrheal Activity of Ethanolic Extract of Leaves of *Barringtoniaacutangula* (L) Gaertn. Inventi Journals (P) Limited.

PearseA.G.E. (1968). Histochemistry: Theoretical and applied. 3rd edition Vol I, Churchill Livingstone, Edinburgh, London, New York.

Peña-Llopis S., Ferrando M.D., Peña J.B. (2003). Fish tolerance toorganophosphate-induced oxidative stress is dependenton the glutathione metabolism and enhanced by N-acetylcysteine.
Aquatic Toxicology. 65:337–360.

Plummer D.T. (1988). An introduction to Practical Biochemistry. Tata-McGraw Hill, New Delhi.

Rahemo Z.I.F., Gorgees N.S. (1987). Studies on the nervous system of *Polystomaintegerrimum* as revealed by acetylthiocholine activity. Parasitology Research. 73: 234-239.

Rahman M.M., Polfreman D., MacGeachan J., Gray A.I. (2005). Antimicrobial activities of *Barringtoniaacutangula*. PhytotherapyReasearch. 19: 543-545.

Rao S. (1925). The colorimetric determination of phosphorus. Journal of Biological Chemistry. 66: 375-400.

Sahoo S., Panda P.K., Mishra S.R., Parida R.K., Ellaiah P., Dash S.K. (2008). Antibacterial activity of *Barringtoniaacutangula* against selected urinary tract pathogens. Indian Journal of Pharmaceutical Sciences.70: 569-677.

Salter M., Knowles R.G. (1998). Measurement of NOS activity by conversion of radiolabelled arginine to citrulline using ion-exchange separation. In: *Nitricoxideprotocols*pp 67-74 Titheradge, MA (ed.). Humana Press, New Jersey, USA.

Schmahl, G., Melhorn, H. (1988) Treatment of fish parasites.4. Effects of sym. triazinone (toltrazuril) on Monogenea. Parasitology Research. 75: 132-145.

Schmahl G., Taraschewski H., Mehlhorn H. (1989). Chemotherapyof fish parasites. Parasitology Research. 75:503–511.

Silva L.S., Parodi T.V., Reckziegel P., Garcia V.O., Bürger M.E., Baldisserotto B., Malmann C.A., Pereira A.M.S., Heinzmann B.M.(2012). Essential oil of *Ocimumgratissimum* L.:anesthetic effects, mechanism of action and tolerance in silver catfish, *Rhamdiaquelen*. Aquaculture. 350–353: 91–97.

Tandon V., Pal P., Roy B.; Rao H.S.P., Reddy K.S. (1997). In vitro anthelmintic activity of root tuber extract of Flemingiavestita, an indigenous plant in Shillong, India. Parasitology Research. 83: 492-498.

Tandon V.,Kar, P.K., Saha N. (2001). NO nerves in trematodes, too! NADPH-diaphorase activity in adult*Fasciolopsisbuski*. Parasitology International. 50(3): 157-163.

Tu X., Ling F., Huang A., Zhang Q.Z., Wang G.X. (2013). Anthelmintic efficacy of *Santalum album* (Santalaceae) against monogenean infections in goldfish. Parasitology Research. 112:2839–2845.

Wachstein M., Meisel E. (1957). Histochemistry of hepatic phosphatase at a physiologic pH..Animal Journal of Clinical Pathology. 27: 13-23.

Wang G.X., Zhou Z., Cheng C., Yao J.Y., Yang Z.W.(2008). Osthol and isopimpinellin from *Fructuscnidii* for the control of*Dactylogyrusintermedius* in *Carassiusauratus*. Veternary Parasitology. 158:144–151.

Wang G.X., Han J., Feng T.T., Li F.Y., Zhu B. (2009). Bioassay-guided isolation and identification of activecompounds from *Fructusarctii* against *Dactylogyrusintermedius* (monogenea) in goldfish (*Carassiusauratus*). Parasitology Research. 106:247–255.

Wang G.X., Zhou Z., Jiang D.X., Han J., Wang J.F, Zhao L.W., Li J.(2010). In vivo anthelmintic activity of five alkaloids from *Macleayamicrocarpa*(Maxim) Fedde against *Dactylogyrusintermedius*in *Carassiusauratus*. Veterinary Parasitology. 171:305–313.

Wang K.Y., Yao L., Du Y.H., Xie J.B., Huang J.L., Yin Z.Q.(2010). Anthelmintic activity of the crude extracts, fractions, and osthole from *Radix angelicaepubescentis* against *Dactylogyrusintermedius* in goldfish (*Carassiusauratus*) in vivo. Parasitology Research. 108: 195–200.

Yadav A.K.(2012). In vivo anthelmintic activity of *Clerodendrumcolebrookianum*Walp., a traditionally used taenicidal plant in Northeast India. Parasitology Research. 111: 1841-1846.

Yao J.Y., Zhou Z.M., Pan X.Y., Hao G.J., Li X.L., Xu Y., Shen J.Y., Ru H.S., Yin W.L. (2011). In vivo anthelmintic activity of chelidonine from *ChelidoniummajusL*. against *Dactylogyrusintermedius*in *Carassiusauratus*. Parasitology Research. 109:1465–1469.

.